Supplementary Materials for

25-Hydroxycholesterol suppresses interleukin-1–driven inflammation downstream of type I interferon

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Materials and Methods

Mice. Wild-type and Ly5.2 (CD45.1) congenic C57BL/6 (B6) mice, 6–12 weeks old, were from National Cancer Institute. *Gpr183-/-* mice (42) were backcrossed to C57BL/6J for 11 generations. These mice carry an eGFP gene inserted in place of the Gpr183 open reading frame. *Ch25h-/-* mice (15) were backcrossed to C57BL/6J for 10 generations. *Nr1h2*$^{+/-}$, *Nr1h23*$^{+/-}$, and *Ifnar1-/-* mice were from Jackson laboratories. Animals were housed in a specific pathogen–free environment in the Laboratory Animal Research Center at UCSF and all experiments conformed to the ethical principles and guidelines approved by the UCSF Institutional and Animal Care and Use Committee.

Cells. Bone marrow (BM) cells were differentiated in DMEM with 10% FBS and 10% M-CSF-conditioned medium for 6–7 days, then plated at 1 x 10^6 cells/ml and cultured overnight. BMDMs were primed with 100 ng/ml LPS (E. coli 0111:B4; Sigma) for the indicated time. For ELISA detection of IL1β, IL1α and IL18 and Caspase-1, ATP (Sigma) was added at the concentration of 5 mM for the final 45 min of culture. In one set of experiments nigericin (0.4 mM; Sigma-Aldrich), alum (300 mg/ml; Pierce Biochemicals), poly(dA:dT) (1 mg/10^6 cells; Sigma-Aldrich) and flagellin (1 mg/10^6 cells; a gift from Anthony DeFranco) were added to LPS-stimulated BMDMs for 12 hours in order to activate inflammasomes.

Bone marrow chimeras. Ly5.2 congenic or *Ch25h*$^{-/-}$ mice were lethally irradiated with either 1,100 or 1,300 rad in split doses and reconstituted with 1–3 x 10^6 BM cells from wild-type and *Ch25h*$^{-/-}$ donors. Mice were analyzed 8–12 weeks later.

BMDM transduction. BM cells were differentiated in DMEM with 10% FBS and 10% M-CSF-conditioned medium for 2 days then spin-infected with a retroviral construct expressing Ch25h, Ch25h mut (20), Insig1 or control and an IRES–Thy1.1 cassette as a reporter. In some experiments a retroviral construct expressing ER-Cre and an IRES-hCD4 was used. Two days after the spin infection, Thy1.1 positive cells were FACS sorted and cultured for a further 3 days before performing any experiment. Cells were always cultured in presence of 10% M-CSF-conditioned medium. ER-Cre transduced BMDMs were cultured in the presence of 4-hydroxytamoxifen (1 μM) for 2 days before performing any experiment.

Flow Cytometry. Cells were stained with Abs to TCRγδ (GL3), TCRβ (H57-597), IL17A (17B7), IFNγ (XMG1.2), CD11b (M1/70), Ly6G (1A8), Ly6C (HK1.4), CD115 (AFS98), F4/80 (CI:A3-1), pro-IL1β (NJTEN3), CD4 (RM4-5), CD44 (IM7) and CD25 (PC61) (from Biolegend, BD Biosciences, or eBioscience). Biotin conjugates were detected with streptavidin Qdot605 (Invitrogen). To detect IL17A and IFNγ, cells were stimulated for 4 h with 50 ng/ml PMA (Sigma-Aldrich) and 1 mg/ml ionomycin (EMD Biosciences) in brefeldin A (BD Biosciences). Cells were stained with fixable viability dye (eFluor780; eBioscience) to exclude dead cells then stained for surface antigens, treated with BD Cytofix Buffer and Perm/Wash reagent (BD Biosciences), and stained with anti–IL17A and anti-IFNγ. To detect pro-IL1β cells were treated with BD Cytofix Buffer and Perm/Wash reagent (BD Biosciences), and stained with anti-pro-IL1β. Intracellular caspase-1 activity was analyzed with CaspaLux1 E1D2 kit (OncoImmunin, Inc.) following the manufacturer's protocol.

Cytokine enzyme-linked immunosorbent assays. Supernatants from cultured BMDMs were collected at the times indicated. Enzyme-linked immunosorbent assay (ELISA) antibody pairs used for IL6, IL1β, IL1α and IL18 were as listed below. All ELISA

antibodies were used at 2 mg/ml for capture and 1 mg/ml for detection. Detection antibodies were biotinylated and labelled by streptavidin-conjugated horseradish peroxidase (HRP), and visualized by the addition of Substrate Reagent Pack (R&D). Color development was stopped with 3 M H2SO4. Recombinant cytokines served as standards and were purchased from Peprotech or R&D. Absorbances at 450 nm were measured on a tunable microplate reader (VersaMax, Molecular Devices). Cytokine supernatant concentrations were calculated by extrapolating absorbance values from standard curves where known concentrations were plotted against absorbance using SoftMax Pro 5 software. Capture/detection antibody pairs were as follows: anti-IL1α unconjugated (364/3B3-14) and biotinylated (Polyclonal) from eBioscience; anti-IL1β, unconjugated (B122) and biotinylated (polyclonal); anti-IL18 biotinylated (74) and biotinylated (93-10C) from MBL international; anti-IL6 unconjugated (MP5-20F3) and biotinylated (MP5-32C11).

Sterols. 25-hydroxycholesterol, 7α,25 hydroxycholesterol and cholesterol (Avanti Polar Lipids) were prepared in ethanol as 10 mM stock solutions.

Endotoxic shock model. Mice (8–10 weeks old) were injected i.v. with 50 mg/kg LPS (E. coli 0111:B4; Sigma) and monitored 8 times daily for a total of 6 days. For serum cytokine measurements, a separate cohort of mice received 20 mg/kg LPS and blood was collected 12 h later.

In vivo peritonitis. For neutrophil recruitment in the peritoneum, mice were injected i.v. with 200 μg poly(I:C) followed by a second i.p. injection of 350 mg alum (Pierce) 5 hr later. 12–14 hr after alum injection, mice were sacrificed, peritoneal cavities were lavaged with 6 ml of medium and peritoneal cells were analyzed by FACS.

EAE model. Groups of female mice 8–10 weeks of age were immunized subcutaneously on day 0 with 100 mg MOG(35–55) (Genemed) emulsified in CFA (Chondrex). Pertussis toxin (List Biological Labs) in 100 ul saline was injected intravenously twice, on days 0 and 2, and disease severity was assigned scores.

Th17 priming. Naive CD4+ T cells were first enriched from spleen and peripheral lymph nodes with anti-CD4-coated magnetic beads (Miltenyi Biotec) and then sorted with a FACSAria (BD Biosciences) as CD4+ CD8– CD25- CD44lo CD62Lhi. Naive CD4+ T cells were stimulated with plate-bound anti-CD3 (2 μg/ml) and soluble anti-CD28 (2 μg/ml) for 4 days. For experiments with BMDM supernatants, the T cells were incubated in a 1 in 2 dilution of 12 hr LPS stimulated BMDM culture supernatant that had been centrifuged and 0.2μm filtered and in the presence or absence of TGFβ (1 ng/ml). For conventional Th17 priming, the T cells were cultured in medium containing IL6 (20 ng/ml, Peprotech), TGFβ (1 ng/ml), IL1β (10 ng/ml, R&D Systems), anti-IFNγ (10 μg/ml) and anti-IL4 (10 μg/ml, BD Biosciences).

Listeria infection. For in vivo infection, mice aged 8-10 weeks were infected intravenously with 5 x 10^5 *L. monocytogenes* i.v. for 2 days. For in vitro infection, BMDMs were seeded onto TC-treated dishes, incubated overnight, and infected with *L. monocytogenes* at a multiplicity of infection (MOI) of 10. After 30 min the infected cells were washed, gentamicin (50 μg/ml) treated and the cells and the supernatant were analyzed after 24 or 48 hours.

Quantification of 25-Hydroxycholesterol. BMDM were treated as above, medium was extracted with organic solvents and 25-HC quantified on a 4000 QTRAP liquid chromatography mass spectrometer (Applied Biosystems) as described (15).

RNA Isolation and Real-Time RT-PCR. Total RNA was isolated from ~$5.0x10^5$ BMDMs with the Trizol reagent (Life technology) following the manufacturer's protocol. Real-time PCR was performed using SYBR Green PCR Mix (Roche) and an ABI prism 7300 sequence detection system (Applied Biosystems, Foster city, CA). Hprt mRNA levels were used as internal controls. Primer sequences are shown in Table S1.

RNA-seq and analysis. Bone marrow-derived macrophages ($1x10^7$) were stimulated with 100ng/ml LPS for 8hrs. Cells were then washed 2x with PBS and the cell pellets were snap frozen in liquid nitrogen and stored at -80°C. RNA was extracted using the Qiagen RNeasy Kit. RNA quality was checked using the Agilent 2100 Bioanalyzer (RIN > 9 for all samples). Barcoded sequencing libraries were then generated using 100ng of RNA with the Ovation RNA-seq System V2 and Encore Rapid Library System. The UCSF Human Genetics Core performed next-generation sequencing (Illumina HiSeq 2500) with 100bp paired end reads. Sequences were reported as FASTQ files, which were aligned to the mm9 mouse genome using STAR (Spliced Transcript Alignment to a Reference). Generation of Log2FC values and further analyses were performed using a Bioconductor package on RStudio. SREBP-target and IFN-stimulated gene sets were from The Broad Institute GSEA. Data have been deposited in GEO under accession number GSE58993

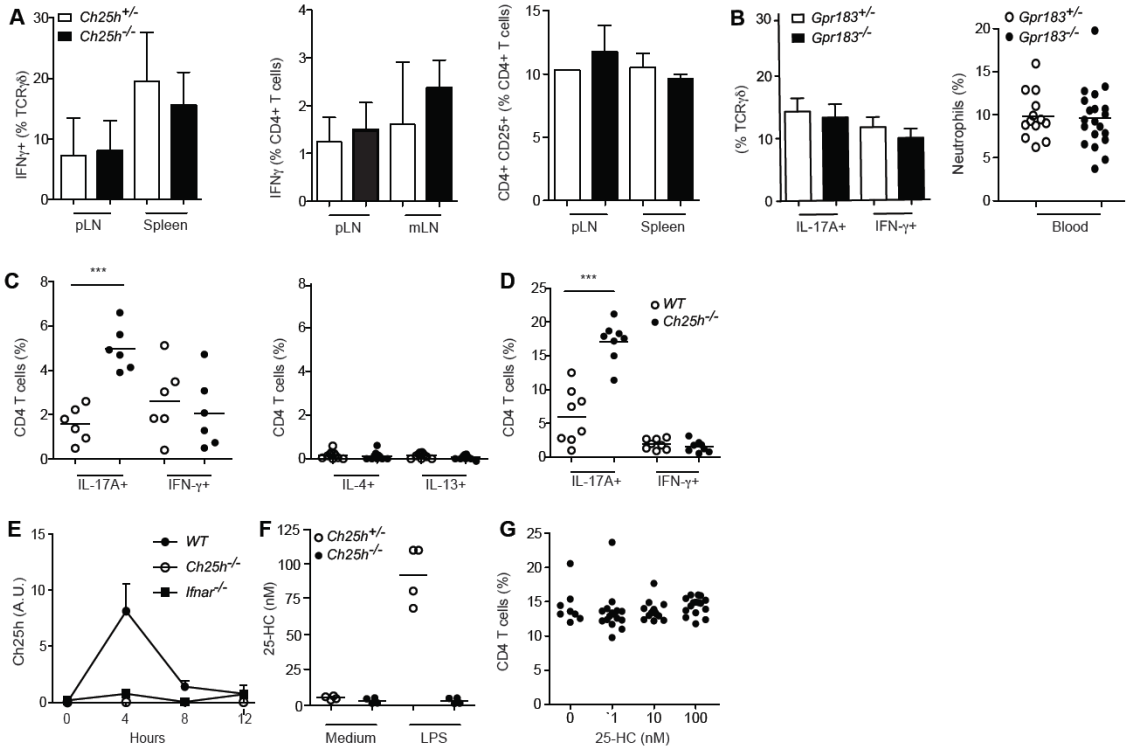

Figure S1. (A) Percent of IFNγ$^+$ γδ T cells, IFNγ$^+$ CD4$^+$ T and CD4$^+$ CD25$^+$ T cells in the indicated organs in *Ch25h*$^{+/-}$ and *Ch25h*$^{-/-}$ mice (n=22 per genotype, mean ± SD)**. (B)** (Left) Percent of IL17A$^+$ and IFNγ$^+$ γδ T cells in pLN in *Gpr183*$^{+/-}$ and *Gpr183*$^{-/-}$ mice (n=13 per genotype, mean ± SD). (Right) Percent of neutrophils in blood of *Gpr183*$^{+/-}$ and *Gpr183*$^{-/-}$ mice (n=13 per genotype, mean ± SD)**. (C)** (Left) Percent of IL17A$^+$ and IFNγ$^+$ CD4 T cells primed *in vitro* with supernatant of LPS-stimulated *Ch25h*$^{+/-}$ or *Ch25h*$^{-/-}$ BMDMs in the absence of TGFβ for 4 days. (Right) Percent of IL4$^+$ and IL13$^+$ CD4 T cells primed *in vitro* with supernatant of LPS-stimulated *Ch25h*$^{+/-}$ or *Ch25h*$^{-/-}$ BMDMs in the presence of 1 ng/ml TGFβ for 4 days. Each point represents cells from an individual mouse and data are pooled from 3 independent experiments. **(D)** Percent of IL17A$^+$ and IFNγ$^+$ CD4 T cells primed in vitro with supernatant of LPS-stimulated *Ch25h*$^{+/-}$ or *Ch25h*$^{-/-}$ peritoneal macrophages in the presence of 1 ng/ml of TGFβ for 4 days. Each point indicates an individual culture and data are pooled from 3 experiments. **(E)** Time course of *Ch25h* mRNA expression in BMDMs generated from the indicated mice and stimulated with LPS (mean ± SD from 6 independent experiments). **(F)** 25-HC concentration in supernatants of control and LPS-stimulated *Ch25h*$^{+/-}$ or *Ch25h*$^{-/-}$ BMDMs. **(G)** Percent of CD4 T cells expressing IL17A following *in vitro* priming under Th17 polarizing conditions and treatment with 25-HC at the indicated concentrations for 5 days. Each point indicates an individual culture and data are pooled from 3 experiments. *, p<0.05; **, p<0.01, ***, p<0.005 (unpaired Student's T-test).

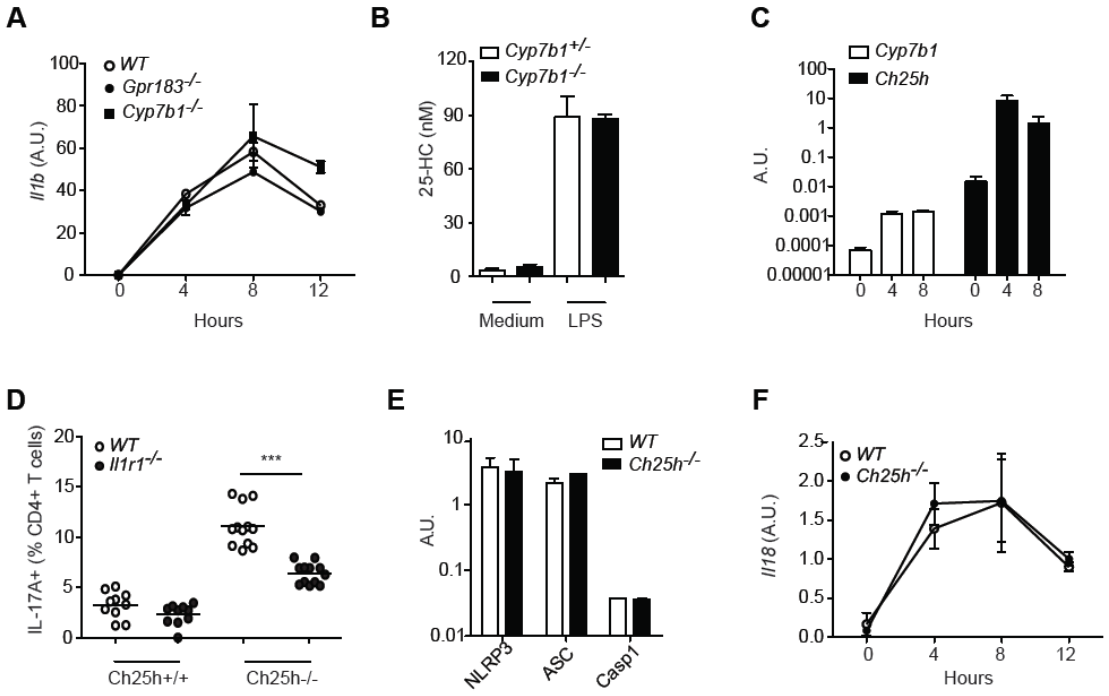

Figure S2. (A) Time course of *Il1b* mRNA expression in BMDMs from wild-type, *Gpr183*$^{-/-}$ and *Cyp7b1*$^{-/-}$ mice stimulated with LPS (mean ± SD from 6 independent experiments). **(B)** 25-HC concentration in supernatant of LPS-stimulated *Cyp7b*$^{+/-}$ or *Cyp7b1*$^{-/-}$ BMDMs (mean ± SD from 3 independent experiments). **(C)** Time course of *Ch25h* and *Cyp7b1* mRNA expression in BMDMs stimulated with LPS. **(D)** Percent of IL17A^{+} CD4 T cells from wild-type or *Il1r1*$^{-/-}$ mice primed *in vitro* with supernatant of LPS-stimulated *Ch25h*$^{+/+}$ or *Ch25h*$^{-/-}$ BMDMs in the presence of 1 ng/ml of TGFβ for 4 days. Each point indicates an individual culture and data are pooled from 3 experiments. **(E)** *Nlrp3, Asc* and *Casp-1* expression in wild-type and *Ch25h*$^{-/-}$ BMDMs stimulated with LPS for 8 hours (mean ± SD from 3 independent experiments). **(F)** Time course of *Il18*m RNA expression in BMDMs from wild-type and *Ch25h*$^{-/-}$ BMDMs stimulated with LPS (mean ± SD from 3 independent experiments). *, $p<0.05$; **, $p<0.01$, ***, $p<0.005$ (unpaired Student's T-test).

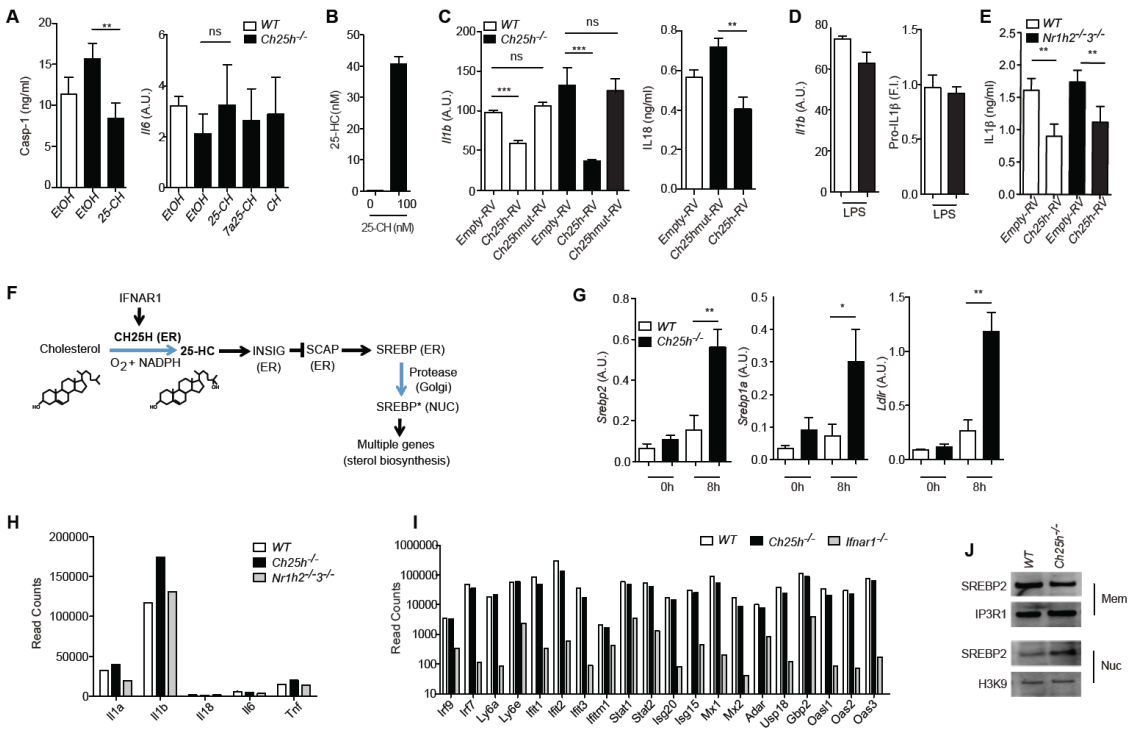

Figure S3. (A) (Left) Secreted caspase-1 in wild-type and *Ch25*$^{-/-}$ BMDMs stimulated with LPS for 8 hours in the presence of 100nM 25-HC or with carrier. Secreted caspase-1 was measured after incubation with ATP. (Right) *Il6* expression in wild-type and *Ch25*$^{-/-}$ BMDMs stimulated with LPS for 8 hours in the presence of 100nM 25-H(C) 7α,25-HC or cholesterol (CH) or with carrier (mean ± SD from 3 independent experiments). **(B)** 25-HC concentration in cell pellet of LPS-stimulated *Ch25h*$^{-/-}$ BMDMs incubated with 100nM 25-HC for 8 hours. (mean ± SD from 3 independent experiments). **(C)** (Left) *Il1b* in BMDMs retrovirally transduced with empty vector, vector encoding Ch25h or mutated Ch25h and stimulated with LPS for 8 hours. (Right) IL18 in BMDMs retrovirally transduced with empty vector, vector encoding Ch25h or mutated Ch25h and stimulated with LPS for 8 hours and ATP for 45 min (mean ± SD from 3 independent experiments). **(D)** *Il1b* and pro-IL1b intracellular level in wild-type and *Nr1h2*$^{-/-}$*3*$^{-/-}$ BMDMs stimulated with LPS for 8 hours (mean ± SD from 3 independent experiments**)**. **(E)** IL1β secretion by wild-type and *Nr1h2*$^{-/-}$*3*$^{-/-}$ BMDMs retrovirally transduced with empty vector or vector encoding Ch25h and stimulated with LPS for 8 hours and ATP for 45 min (mean ± SD from 3 independent experiments). **(F)** Pathway of 25-HC induction and repressive action on sterol biosynthesis. CH25H, INSIG, SCAP and SREBP are each transmembrane proteins situated in the endoplasmic reticulum (ER). IFNAR1 signaling upregulates CH25H, generating 25-HC that binds INSIG and causes it to inhibit SCAP and prevent SREBP leaving the ER. When 25-HC is low, INSIG releases SCAP and allows it to chaperone SREBP to the Golgi where it undergoes proteolytic processing, leading to release of the active form (SREBP*) for movement to the nucleus (NUC) to act as a transcription factor. **(G)** *Srebp1a Srebp2* and *Ldlr* transcript expression determined by QPCR in wild-type and *Ch25h*$^{-/-}$ BMDMs stimulated with LPS for 8 hours (mean ± SD from 3 independent experiments). **(H)** RNAseq data for inflammatory

cytokines from 8 hour LPS-stimulated wild-type, *Ch25h*$^{-/-}$ or *Nr1h2*$^{-/-}$*3*$^{-/-}$ BMDMs**.** Bars indicate means (n=2). **(I)** RNAseq data for IFN-stimulated genes from 8 hour LPS-stimulated wild-type, *Ch25h*$^{-/-}$ or *Ifnar1*$^{-/-}$ BMDMs**.** Bars indicate means (n=2). **(J)** Immunoblotting for membrane resident and nuclear SREBP2 and loading controls (IP3R1 and H3K9) on membrane and nuclear fractions from wild-type and *Ch25h*$^{-/-}$ BMDMs stimulated with LPS for 8 hours.

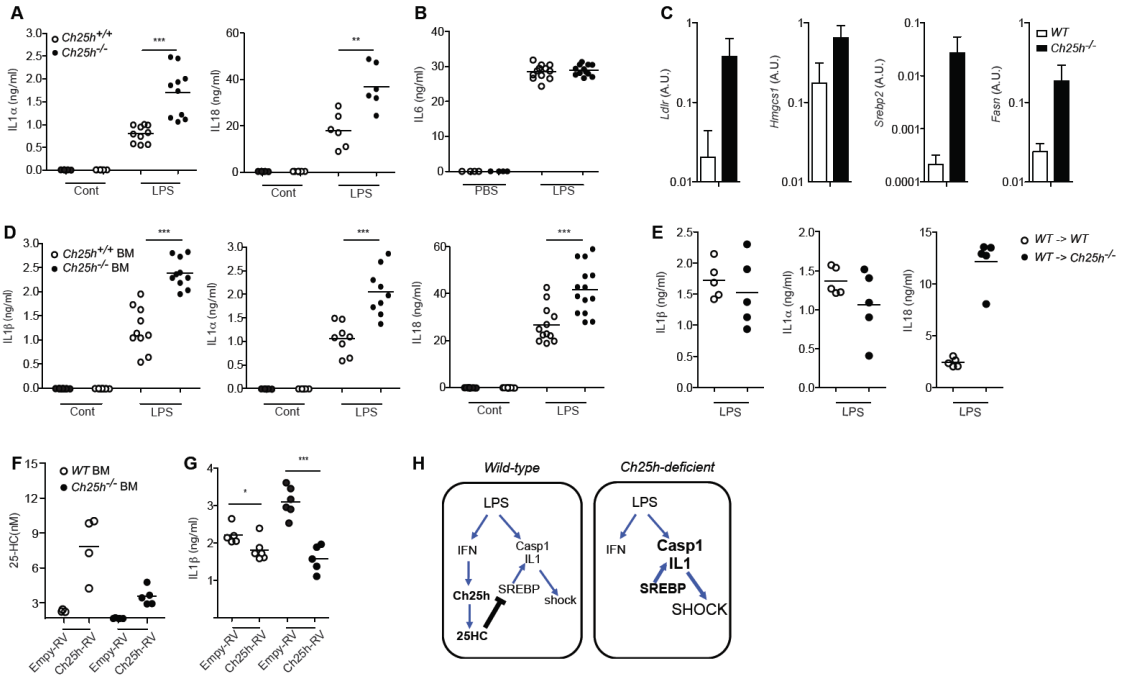

Figure S4. **(A)** Serum IL1α and IL18 in *Ch25h*$^{+/-}$ and *Ch25h*$^{-/-}$ mice 12 hours after injection of 20 mg/kg LPS. **(B)** Serum IL6 in wild-type and *Ch25h*$^{-/-}$ mice 12 hours after injection of 20 mg/kg LPS. Each point in A and B represents an individual mouse and data are pooled from 3 experiments. **(C)** *Ldlr*, *Hmgcs1, Srebp2* and *Fasn* transcript expression determined by QPCR in splenic macrophages from wild-type and *Ch25h*$^{-/-}$ mice treated 6 hours earlier with 20 mg/kg LPS (mean ± SD, n=3). **(D)** Serum IL1β, IL1α and IL18 12 hours after injection of 20 mg/kg LPS in wild-type mice irradiated and transplanted with wild-type or *Ch25h*$^{-/-}$ BM for 8 weeks. Cont indicates uninjected control chimeras. Each point represents an individual mouse and data are pooled from 3 experiments. **(E)** Serum IL1β, IL1α and IL18 12 hours after injection of 20 mg/kg LPS in wild-type or *Ch25h*$^{-/-}$ mice irradiated and transplanted with wild-type BM for 8 weeks. Each point represents an individual mouse and data are pooled from 2 experiments. **(F)** 25-HC concentration in serum of mice irradiated and transplanted with wild-type or *Ch25h*$^{-/-}$ BM retrovirally transduced with either empty vector or vector encoding Ch25h **(G)** Serum IL1β 12 hours after injection of 20 mg/kg LPS in mice in F. Each point in F and G represents an individual mouse and data are pooled from 2 experiments. **(H)** Model for 25-HC induction and repressive action during LPS-induced septic shock.

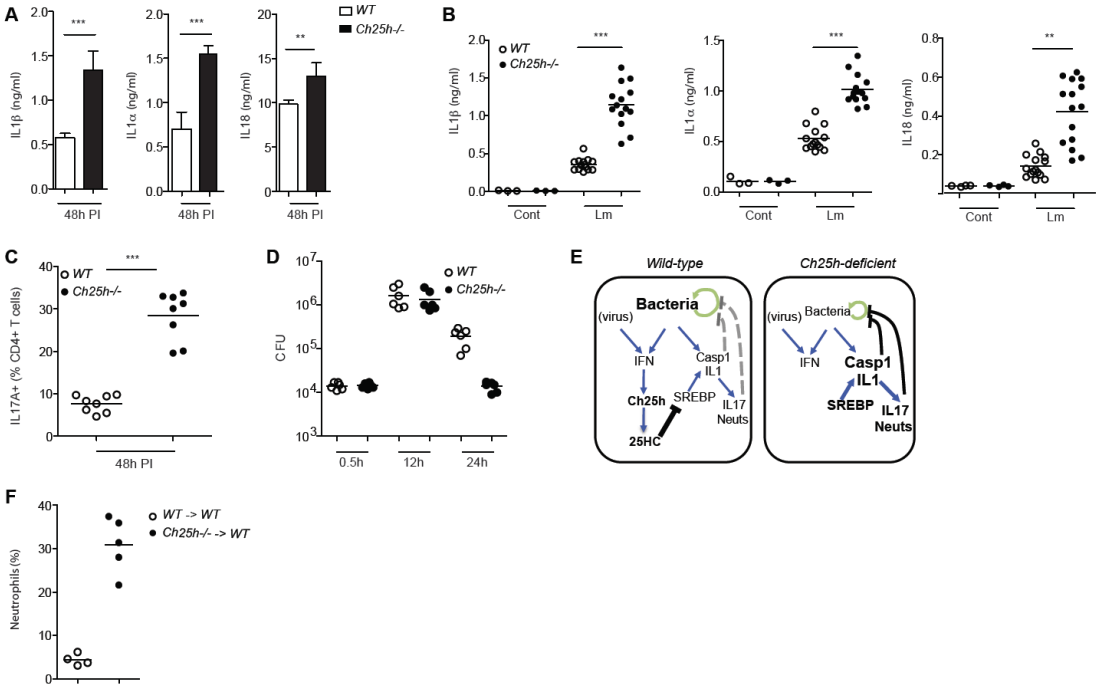

Figure S5. (A) Serum IL1β, IL1α and IL18 from mice infected with $5x10^5$ *L. monocytogenes* for 3 days (mean ± SD from 3 independent experiments). **(B)** IL1β, IL1α and IL-18 secreted by BMDMs cultured with *L. monocytogenes* for 48 hours (MOI=10). Each point represents an individual mouse and data are pooled from 3 experiments. **(C)** Percent of $IL17A^+$ CD4 T cells primed for 4 days *in vitro* with supernatant of BMDMs that had been cultured with *L. monocytogenes* for 48 hours (MOI=10). Each point represents an independent BMDM culture and data are pooled from 3 experiments. **(D)** Intracellular growth of *L. monocytogenes* in wild-type or $Ch25h^{-/-}$ BMDMs. Each point represents an independent BMDM culture and data are pooled from 2 experiments. **(E)** Model for 25-HC induction and repressive action during a type I IFN-inducing bacterial infection or bacterial and viral co-infection. Neuts, neutrophils. **(F)** Frequency of neutrophils in the peritoneal cavity of BM chimeric mice challenged with Poly(I:C) followed by Alum as in Fig. 4E. Each point represents an individual mouse and data are pooled from 2 experiments.*, $p<0.05$; **, $p<0.01$, ***, $p<0.005$ (unpaired Student's T-test).

Table S1.

	Forward primer	**Reverse primer**
Hprt	AGGTTGCAAGCTTGCTGGT	TGAAGTACTCATTATAGTCAAGGGCA
Il1b	AGCTTCCTTGTGCAAGTGTCT	GACAGCCCAGGTCAAAGGTT
Il6	TGGCTAAGGACCAAGACCATCCAA	AACGCACTAGGTTTGCCGAGTAGA
Il18	TCAAAGTGCCAGTGAACCCC	GGTCACAGCCAGTCCTCTTAC
Il23p19	TATCCAGTGTGAAGATGGTTGTG	CACTAAGGGCTCAGTCAGAGTTG
Ch25h	GCGACGCTACAAGATCCA	CACGAACACCAGGTGCTG
Cyp7b1	TTCCTCCACTCATACACAATG	CGTGCTTTTCTTCTTACCATC
Abcg1	TTCCCCTGGAGATGAGTGT	CAGTAGGCCACAGGGAACAT
Nlrp1a	AGGCTCTTTACCCTCTTCTA	ATGTGCTTCTTCTTCTGGTA
Nlrp3	TCCTGCAGAGCCTACAGTTG	ACGCCTACCAGGAAATCTCG
Nlrc4	TCACCACGGATGACGAACAG	GTCAATCAGACCACCTGGCA
Aim2	ACAAAGGCAGTGGGAACAAGA	GAAAACTTCCTGACGCCACC
Asc	GCCAGAACAGGACACTTTGTG	ACACTGCCATGCAAAGCATC
Caspase1	CGCCCTGTTGGAAAGGAACT	CCCTCAGGATCTTGTCAGCC
Caspase11	ACAATGCTGAACGCAGTGAC	CTGGTTCCTCCATTTCCAGA
Srebp1a	TTGGCACCTGGGCTGCT	GCGCCATGGACGAGCTG
Srebp2	CTTGACTTCCTTGCTGCA	GCGTGAGTGTGGGCGAATC
Ldlr	GAAGTCGACACTGTACTGACCACC	CTCCTCATTCCCTCTGCCAGCCAT
pre-Il1b	GGGCCTCAAAGGAAAGAATC	AAAGGCAGAGTCTTCGGTGA
Fasn	ACCCGAGGGATCTGGTGAA	CTGTCGTGTCAGTAGCCGAG
Hmgcs1	GGAAGCCTTTGGGGACGTTA	ACACTCCAACCCTCTTCCCT